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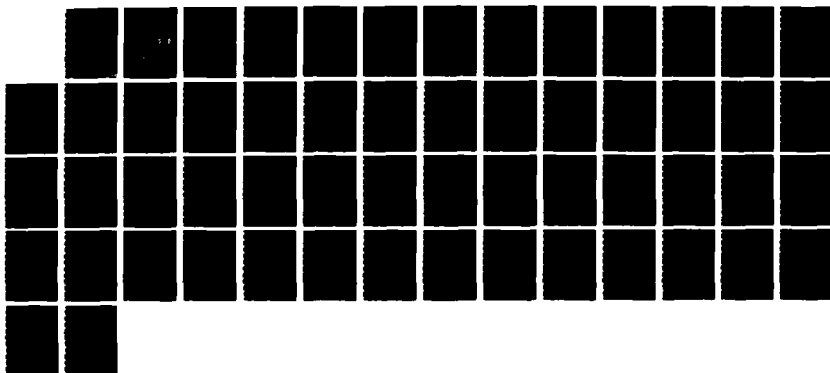
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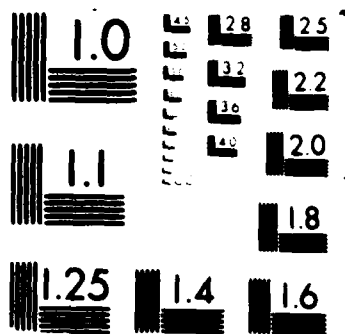
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BIOSYNTHESIS OF BETA-NITROPROPIONIC ACID AND ITS
ESTERIFICATION TO CELLULOSE

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<p>The object of this program was the enzymatic esterification of beta-nitropropionic acid, NPA, to cellulose. A further objective was the enzymatic synthesis of NPA. To achieve these objectives, both natural and semisynthetic enzymes were employed. The achievements under the contract are:</p>					
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ABSTRACT

19. 1. Analysis of the transesterification reaction of cellulose with the beta-nitropropionic acid methyl ester, NPAME, in the presence of lipase indicated that the reaction was indeed catalyzed by the enzyme (see section "Polysaccharide esters", page 12).

2. Enzymatic catalysis of the nitration of propionaldehyde (see section "Nitration", page 7).

3. A deliverable consisting of NPA ester of alpha-methyl-D-glucoside was prepared by organic synthesis techniques (see section "Monosaccharide esters", page 11).

Enzymatic catalysis of the transesterification reaction would fulfill one objective of the contract. When lipase was used as a catalyst, the evidence indicates that some NPA ester of cellulose did indeed form. The concentration of the ester was so low that extraction of this product was not practical.

Enzymatic catalysis of the nitration reaction is a key reaction in the synthesis of NPA. Evidence that peroxidase catalyzes this reaction has also been presented.

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INTRODUCTION

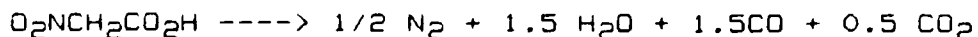
Nitrocellulose has unique properties that make it desirable as a gun propellant. Most of these desirable properties center on the structural integrity of the modified polymer and is, in part, inherited from the hydrogen bonded structure of the base polymer-cellulose. The structure/property relationships wherein degree of nitration and molecular weight ranges were the controlled variables have been reported (1-2).² In addition to the hydrogen bonding having an effect on physical structure, residual cellulosic hydroxyl groups act as an hydration locus which lowers the thermal and mechanical sensitivity of the material.

The production of nitrocellulose, as with most explosives and propellants, is an energy intensive process since the components of the mixed acid nitration mixtures are the products of either high pressure technology (ammonia production) or require special materials for synthesis and storage (3).² *Key word: propellant* →

The nitro group is not one that is found widely in natural products. Indeed organic nitro compounds are usually the result of industrial organic chemistry. Therefore the incorporation of the nitro group in an organic compound as a result of a natural biosynthesis is unusual. The biosynthesis of beta-nitro-propionic acid (NPA) is one such process, and is discussed further.

The constraints of present and potential national energy curtailments and the greater awareness of the commercial use of bioprocesses (because of the "genetic engineering" revolution) has led to considerations of non-classical methods for the preparation of high energy compounds. This is a logical train of thought since bioprocesses are catalyzed by enzymes which usually function best in low temperature regimes (15-40°C), mild pH and aqueous or aprotic solvents.

The derivatives of cellulose prepared by partial esterification with aliphatic acids (cellulose acetate, cellulose propionate, cellulose butyrate etc.) are tonnage commercial plastics. By synthetic analogy, an energetic group could be introduced into the cellulosic macromolecule via esterification with, for example, nitroacetic acid. Nitroacetic acid has excellent oxygen balance as can be seen by the theoretical decomposition reaction:

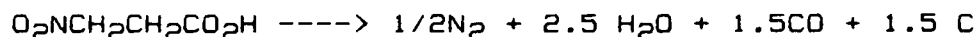


Unfortunately nitroacetic acid is unstable, undergoing decarboxylation to yield nitro methane (4).

This reaction also occurs with other alpha-nitroacids. Thus attempts to prepare 2-nitropropionic acid by straightforward means resulted in the production of nitroethane (5).

Beta-nitroaliphatic acids do not undergo this reaction and

hence they can be derivatized. While 3-nitropropionic acid does not have an outstanding oxygen balance;



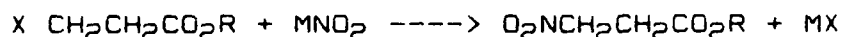
it can still serve as a model for esterification studies of cellulose.

Synthesis of B-NPA

In the following subsections the classical organic synthesis, the known microbial synthesis and a proposed new route using extra-cellular enzymes for B-NPA synthesis are discussed.

Organic synthesis

The preparation of nitro aliphatic acid has been reviewed by Kornblum (6) as well as Buehler and Pearson (7). The only practical method reported for the strictly classical organic synthetic preparation of 3-nitropropionic acid (or its esters) is by metathesis using the corresponding haloacid;



where X is usually either chloro or bromo, M is either silver, sodium or potassium and R is either H (the free acid) or an alkyl group (the simple esters). 3-Nitropropionic acid is listed by Aldrich (N-2,290-8) and Aldrich states that their material is prepared from the bromoacid and potassium nitrate (Private communication).

Microbial synthesis

The biosynthesis of nitro compounds has been reported relatively rarely in the literature (8-12). Paul Shaw and his co-workers have studied the biosynthesis of NPA by Penicillium atrovenetum. Yields of NPA based on glucose as a carbon source range from less than 1% to 3.3%. The proposed synthesis route is:



NPA as well as glucose esters of NPA have been isolated from crownvetch (13).

Enzymatic method

Direct enzymatic synthesis. Enzymatic methods offer the advantages of superior reaction rates, mild conditions, and selectivity of reaction. The same enzymes that catalyze the hydrolysis of esters are also capable of ester synthesis under suitable reaction conditions. However, when the reaction is performed in aqueous medium, the yield of ester is often very low, because at equilibrium most of the ester is hydrolyzed. An organic solvent medium is often not compatible with enzyme

stability. Many approaches have been adopted to circumvent such problems, and to optimize the yield of ester.

These include: a. use of a medium consisting of high organic and low water content to shift the equilibrium to ester synthesis; b. use of immobilized esterases, which may have greater stability in an organic medium; c. biphasic medium consisting of water/water-immiscible organic solvent.

Esterase-catalyzed ester synthesis can be performed by two processes: a. esterase catalyzed condensation of acid and alcohol, which is the direct reversal of esterolysis and b. by transesterification, where an alcohol is used as the acyl-acceptor in place of water during esterolysis.

Chymotrypsin catalyzes the esterification of N-acetyl tyrosine with ethanol (14) in a 1:1 mixture of ethanol and glycerol, the latter reagent being added to stabilize the enzyme. The optimal conversion of the acid to ester was 30% at a water concentration of 50%. At higher water concentration, the hydrolysis of the ester was favored, while at lower water content, the rate of synthesis was low, possibly owing to the inactivation of the enzyme. With chymotrypsin immobilized on carboxymethyl cellulose, the synthesis of ester proceeded at water concentration as low as 20%, and the equilibrium concentration of ester reached nearly 40%. Using subtilisin as the catalyst, the esterification of N-acetyl tyrosine reached at least up to 50% in a reaction medium containing 5-10% water (14).

Immobilized tannase from Aspergillus niger generates gallic acid esters with alcohols of chain lengths C-1 to C-12, when incubated with a solution of gallic acid in the appropriate alcohol (15). Ester synthesis can thus be effectively achieved by reversal of enzymatic esterolysis in a suitable reaction medium.

Hydrolysis of esters by many natural esterases involves the intermediate formation of an acyl-enzyme, from which the acyl group is transferred to water, thereby producing the acid product. A classical example is the hydrolysis of esters by serine proteases- chymotrypsin and trypsin (16). Acyl-enzyme intermediates have also been demonstrated for carboxylic acid esterases (17). Most hydrolases can use acceptors other than water, such as alcohols and amines. When an alcohol is used as the acceptor instead of water, the resulting reaction products will include the ester of the acceptor alcohol.

Carboxylesterase of porcine liver hydrolyzes phenylacetate, and generates methyl acetate, in the presence of methanol (18). Deacylation of trans-cinnamoyl-alpha-chymotrypsin in methanol-water solution has been shown to generate methyl cinnamate and cinnamate ion, by spectrophotometric studies (19). Wang et al. have explored the kinetics of chymotrypsin-catalyzed transesterification between p-nitrophenyl-2(-5-N-alkyl) furoate and various alcohol-acceptors.

Generation of new esters has been demonstrated by gas-liquid chromatography (20).

Formation of new esters by means of an enzymatic transesterification reaction has also been demonstrated using porcine pancreatic lipase (21). This enzyme normally hydrolyzes tributyril glycerol to produce glycerol and butyric acid. When the reaction is conducted in the presence of alcohol, the formation of the corresponding butyric acid ester has been demonstrated by gas chromatography. Transesterification, using an alcohol as acceptor during an esterase reaction, offers an alternate method for the preparation of esters.

Biphasic systems. When the reactions are carried out in a medium containing water and water-miscible organic solvents, neither the reversal of enzymatic hydrolysis, nor the transesterification, is ideal for large scale synthesis of esters. While high concentrations of water in the reaction medium shifts the equilibrium strongly towards the starting reagents, performing the reaction in organic solvent medium results in the inactivation of enzymes, due to partial denaturation.

An approach to circumvent this predicament has been developed by Klibanov et al (21-23). A biphasic system consisting of "water and a water-immiscible organic solvent" is used as the reaction medium. In such a system, the enzyme is confined to the aqueous phase, because of its superior solubility in water. Substrates, which are dissolved in the organic phase, freely diffuse into the aqueous phase where the ester formation is catalyzed by the enzyme. The ester thus formed diffuses back to the organic phase and thus is protected from aqueous hydrolysis.

Nearly 100% conversion of N-acetyl tryptophan to its ethyl ester has been reported using chymotrypsin, in a water-chloroform biphasic system (22). Chymotrypsin, immobilized on porous glass, either covalently or non-covalently, is used to prevent the denaturation of the enzyme in aqueous-organic interphase.

Effective use of biphasic solvent systems for esterase catalyzed transesterification has also been described (23). Hog liver carboxyesterase, entrapped into Sepharose or Chromosorb, is used as the catalyst. Reactants, methyl propionate and water immiscible alcohols, constitute the organic phase. Successful synthesis of several optically active alcohols and their propionate esters in gram quantities has been achieved by this method.

Transesterification in biphasic system has also been demonstrated using another esterase, namely, lipase from yeast (23). This enzyme, unlike the carboxyl esterase, has wide nucleophile specificity, and hence a wide variety of acceptor alcohols can be used for transesterification. The aqueous phase consisted of lipase confined to the pores of Chromosorb, and the

organic phase was the acceptor alcohol containing the natural substrate of lipase, tributyrin or glycerol tributyrate. Yields of the new ester produced ranged from 44-90% depending on the nature of the acceptor alcohol.

Use of esterases in a biphasic system thus offers an efficient approach to prepare quantitative amounts of esters, either by reversal of esterolysis or by transesterification.

Semisynthetic enzymes

Even though enzymes offer many advantages over chemical catalysts, use of natural enzymes for commercial processes is not always practical. An enzyme needed to catalyze a desired reaction may not always be found in nature. If an enzyme exists in trace amounts, then the process of isolation and purification can make the enzyme too expensive. Moreover, highly purified enzymes are often not very stable. In addition, the kinetics of an enzyme may not always be compatible for commercial use. Under these circumstances, when the use of a natural enzyme is not practical, semisynthesis of the desired enzyme may be a reasonable alternative.

Methods available

Semisynthesis of an enzyme, as the name implies, involves the use of a naturally available protein or polypeptides as the starting material to generate a protein catalyst. Many methods have been adopted to make a semisynthetic enzyme.

A classical method of enzyme semisynthesis consists of binding a synthetic model peptide to an inactive polypeptide fragment of a natural enzyme. The binding is achieved either non-covalently, or by covalent linkages such as peptide bonds or disulfide linkages (24-25). The semisynthetic enzymes prepared by this method will generally possess the catalytic activity of the enzyme whose polypeptide was used as the starting material. This type of enzyme semisynthesis is particularly useful in studying the kinetic mechanisms of natural enzymes.

Genetic engineering has also been applied to prepare semisynthetic enzymes (26,27). The use of modified DNA allows the synthesis of enzymes possessing different amino acid sequences. Changes in the amino acid residues in the active site allows one to study the structure-function relationships, and may also change the catalytic properties of the protein. This method is more versatile than the peptide attachment method, but involves high experimental cost.

New catalytic activities can be induced in proteins by means of semisynthesis. Kaiser et al (28-31) have generated oxido-reductase activity, by covalently attaching flavin derivatives to the active-site of papain. Hydrogenase activity has been generated from bovine serum albumin, by binding with iron clusters (32). Hemoglobin, when cross-linked with

glutaraldehyde has been shown to acquire hydroxylase activity (33). A novel, systematic approach, developed at Owens-Illinois, Inc. by the researchers who are now employed by Anatrace, Inc. can be applied to prepare semisynthetic enzymes (34-37). This method is discussed in detail in the following section.

Method developed at Owens-Illinois, Inc.

General method. The method invented by Dr. Keyes for preparing semisynthetic enzymes consists of perturbing the natural conformation of a protein, and subsequent binding of a competitive inhibitor of a model enzyme whose activity we wish to mimic. Our process involves three steps:

- a. A commercially available protein is chosen as the starting material. Conformation of the protein is perturbed by changing the pH, temperature, ionic strength, or other conditions.
- b. The perturbed protein is then placed in contact with a modifier.
- c. The newly formed conformation is preserved by cross-linking.

The modifier, and the excess cross-linking reagent is removed by dialysis or gel-filtration.

We have successfully prepared several semisynthetic enzymes by this approach.

Semisynthetic esterases. An ongoing DOE project (DE-AC02-81-ER12003) involves the application of this method to the preparation of semisynthetic esterases and to the investigation of the nature of these semisynthetic enzymes. Dr. Keyes and Dr. Vasan have used bovine pancreatic ribonuclease (RNase) as the starting material for the preparation of semisynthetic esterases. The structure of RNase is perturbed by exposing to acidic conditions. Indole propionic acid, a known competitive inhibitor of chymotrypsin, is used as the modifier. The modified conformation is retained by cross-linking with glutaraldehyde. Modification of RNase by our procedure has generated two semisynthetic esterases, an acid-esterase with optimal pH of 6.0, and a neutral-esterase which has a pH optimum of 7.5 (36,37).

The semisynthetic esterases can be treated like natural enzymes during purification. Classical fractionation techniques such as salting out and molecular sieving have been effectively used to separate and purify these esterases. Nearly 100 fold purification of both the esterases has been achieved by means of ammonium sulfate fractionation and gel filtration on Biogel P-30. The purified esterases possessed Michaelis-Menten kinetics.

OBJECTIVES OF RESEARCH PROGRAM

The objective of this program was the enzymatic esterification of beta-nitropropionic acid (NPA) to cellulose. A further objective was the investigation of the biosynthesis of NPA.

BIOSYNTHESIS OF BETA-NITROPROPIONIC ACID

The synthesis of NPA was explored by two different routes. One method was to investigate the oxidation of primary amines to nitro groups while the other method was an effort to incorporate a nitro group into simple aliphatic compounds.

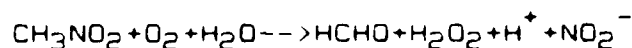
Oxidation of beta-alanine

Initially, oxidation of beta alanine was attempted by chemical means. The oxidation of the amine group of beta-alanine to a nitro group should result in the appearance of an absorbance band in the ultraviolet at approximately 265 nm. The mixing of a solution of beta-alanine with hydrogen peroxide does result in an increase of absorbance at 265 nm. After several hours the reaction appears complete and the absorbance at 265 nm is 1.17.

Since hydrogen peroxide appeared to oxidize beta-alanine, the oxidation was performed again in the presence and absence of peroxidase. Table 1 shows the absorbance values at 265 nm for the samples (beta-alanine, peroxidase, H_2O_2) and the values for the combined controls (Control A: beta-alanine, H_2O_2 and Control B: beta-alanine, peroxidase) (last two columns). The combined control value is compared to the sample absorbance. A greater absorbance for the sample than for its combined controls would indicate production of NPA. Table 1 illustrates that each sample has an absorbance within error of its combined controls. Therefore peroxidase does not appear to be a suitable candidate for the enzymatic oxidation of beta-alanine.

Nitration

The synthesis of nitropropionic acid by nitration was also investigated. D-amino acid oxidase is known to oxidize nitroethane by the following reaction:



Enzymes that can catalyze this reaction may also be able to catalyze the oxidation of nitropropionic acid. Since catalysis is reversible, one would expect that nitropropane could be formed from nitrite, hydrogen peroxide, and propionaldehyde. Three enzymes (glucose oxidase, D-amino acid oxidase, and horse radish peroxidase) were chosen as catalysts because of their ability to oxidize nitroalkanes (38).

Glucose oxidase was studied first for its catalytic activity towards the oxidation of the simple nitroalkanes, nitroethane and nitropropane, and then for its catalytic activity towards the oxidation of nitropropionic acid. The activity was measured in terms of micromoles H_2O_2 produced per gram glucose oxidase, and the values obtained for the various reactions are shown in table 2. From table 2, we see that the order of activity for glucose oxidase oxidation is as follows:

Nitroethane > nitropropane > nitropropionic acid.

It was observed that when D-amino acid oxidase is added to a solution of H_2O_2 , the H_2O_2 concentration decreases (see experimental section) which makes the measurement of D-amino acid oxidase activity towards the oxidation of nitroalkanes difficult.

Horse radish peroxidase was also tested for its ability to oxidize nitropropane to propionaldehyde and HNO_2 . By monitoring HNO_2 production, an activity of $1.7 \pm .7$ U/gram was obtained. Measurement of the reverse reaction was accomplished by observing the decrease in nitrite and was calculated to be 170 ± 60 units/gram at pH 6.9. The concentration of nitrite is measured by an Orion nitrite electrode. No activity was observed at other pH values (5.8, 6.2 and 7.8).

ESTERIFICATION

Measurement of hydrolysis

Non-enzymatic

Figure 1 shows the percent hydrolysis of a commercial sample of ENA (ethyl nitro acetate). Hydrolysis was monitored by measuring micromoles of ethanol generated with Sigma's diagnostic kit # 332-UV (see experimental section). One mole of ethanol is produced for every mole of ENA hydrolyzed. Percent hydrolysis is expressed as the mole percent of ethanol produced per mole of ENA. A higher hydrolysis rate occurs at both alkaline and acid pH compared to neutral pH. The greatest rate of hydrolysis occurs at pH 10.0. The rates of hydrolysis are all relatively the same regardless of buffer selected.

Natural enzyme hydrolysis

There are numerous techniques which can be used to analyze ester hydrolysis. The two methods which we developed to monitor the hydrolysis of nitropropionic acid esters are high performance liquid chromatography (HPLC) and a spectrophotometric assay utilizing the enzymatic oxidation of ethanol. The alcohol oxidase and related reagents are purchased from Sigma Chemical Company in kit form. These methods are discussed in further detail in the experimental section.

Table 3 lists activity measurements (units/gram) obtained for natural enzymes when various nitro esters were used as substrates. When shown, the error values denote the possible error of activity using linear regression. The table also lists the pH values for the solutions in which these reactions were carried out and the technique used to monitor the reaction. Activities of 829 and 1980 units per gram are listed for the hydrolysis of ENA with carboxyl esterase at pH 7.0. This discrepancy may be due to solution composition. The value of 829 U/G was obtained using potassium phosphate buffer, and the value of 1980 U/G was obtained using tris acetate as a buffer.

It is reported in the literature that carboxyl esterase kinetic mechanism does not follow the Michaelis-Menton equation (39) which could explain the variation of activities obtained for the hydrolysis of NPAEE at pH 7.3. Figure 2 shows a plot of carboxyl esterase concentration versus activity (monitoring NPA produced) and from this figure, we see that as the carboxyl esterase concentration increases specific activity decreases. For typical enzyme kinetics, the specific activity (U/g) should not change with changes in enzyme concentration.

Table 4 shows variation in activities obtained for the two different techniques (1) monitoring NPA and 2) monitoring NPAEE). The NPAEE was purified by applying to a fractogel (R) column which removes any excess NPA. Upon purification the activities calculated by both NPA and NPAEE monitoring are similar (table 4).

Semisynthetic enzyme hydrolysis

Table 5 lists activity measurements obtained with semisynthetic enzymes. The table also shows the enzyme, modifier and bifunctional reagent used to prepare the semisynthetic enzyme. Only those modified enzymes from table 6 are listed that were calculated to have activities 0.5 u/g above the error determined by linear regression. The methods for preparing these semisynthetic enzymes and the techniques used to study their hydrolysis with esters are discussed in the experimental section.

Although several preparations using ribonuclease and ficin were active, the specific activity was only one or two units per gram. At this level, the activity is difficult to measure and thus semisynthetic enzymes were not tested for their ability to synthesize NPA esters in mixed solvents.

Enzymatic synthesis of esters

NPA was reacted with both 1-butanol and isopropanol in the presence of lipase to attempt the synthesis of the esters corresponding to the two alcohols. The reaction samples were monitored by HPLC (experimental section) and neither reaction showed evidence of ester formation.

NPA was also reacted with methanol in the presence of

carboxyl esterase under various conditions (see experimental section) to attempt the synthesis of NPAME, and HPLC was used to monitor this reaction. Although there was evidence of ester formation in the reaction samples, controls consisting of only NPA and methanol showed slightly more ester formation than these samples. So the esterification of NPA to NPAME is not catalyzed by carboxyl esterase under the specific conditions mentioned in the experimental section.

Organic synthesis of esters

Simple esters

The desired route to prepare the methyl, ethyl, isopropyl, and isobutyl esters of nitropropionic acid was by direct esterification. The acid was dissolved in the alcohol corresponding to the desired ester and this solution was refluxed using a catalyst while removing water with a trap containing size 3A molecular sieves. By using a reverse phase C₁₈ column (Baker) with an eluent of 0.01M potassium phosphate buffer, pH 7.3, containing 33% acetonitrile, each ester can be analyzed (figure 3).

Figure 3 confirms the predicted results. Figure 3 shows the chromatogram of a sample containing all four esters plus the free acid. Each ester along with the free acid were passed through column individually to ensure positive identification of the peaks in figure 3. The free acid elutes from the column first because of its negative charge. After the free acid, the esters elute from the column in the following order: 1) methyl 2) ethyl 3) isopropyl and 4) isobutyl.

When each of the four nitropropionic acid esters were applied separately to the HPLC column just mentioned, an acid peak was present in the chromatogram along with the ester peak. Thus, for each ester's individual chromatogram, we can calculate the percentage of total area under both the peaks that corresponds to the ester. If we assume that all four nitropropionic acid esters and the nitropropionic acid have the same extinction coefficients, then the molar percentage of ester in the sample is approximately equal to the percentage of total area under the peaks that corresponds to ester only. Table 7 lists the purity of these four nitropropionic acid esters as expressed in terms of percent ester in the sample as calculated in the manner just mentioned.

Monosaccharide esters

The tetra beta nitropropionic acid derivative of either alpha- or beta-methyl D-glucoside was prepared by three different methods:

1. Using dioxane as a solvent without a catalyst.
2. Using dioxane as a solvent and a strongly acidic

ion exchange resin as a catalyst.

3. Adding the alpha-methyl-D-glucoside to melted NPA and heating the mixture to 85°C under vacuum.

Separation of the products and reactants by gel permeation chromatography indicates that the second and third methods give acceptable yields. Since the third method is the simpler of the two, it was used to prepare the deliverables.

Sephadex G-10 can separate compounds with molecular weights between 100 and 700. Both the starting material and desired product have molecular weights in this range. A sample of NPA starting material and the products were analyzed by dissolving in 0.001M HCl, filtering, and passing through a column packed with Sephadex G-10 gel (150 ml) using .001M HCl as an eluent. Figures 4-7 show the chromatograms obtained from passing samples through this column.

The elution volume of NPA is 160 ml (figure 4). Figure 5 shows the chromatogram of the product from the reaction of nitropropionic acid and beta-methyl-D-glucoside in dioxane without a catalyst. Although there is some indication of the desired product, considerable starting material remains. Figure 6 shows the chromatogram for the reaction of nitropropionic acid and beta-methyl-D-glucoside in dioxane with an ion exchange catalyst present. There are traces of the mono, di and tri substituted derivatives of methyl-D-glucoside, but most of the sample is the desired tetra substituted beta-methyl-D-glucoside derivative. Figure 7 shows the chromatogram of the reaction of NPA and alpha-methyl-D-glucoside without solvent or catalyst, and as in figure 6 the desired product was the largest peak with traces of the lower substituted derivatives and no evidence of starting material. From the areas of the curves in figure 7, the mole percent of ester is approximately 85%.

TRANSESTERIFICATION

Enzymatic synthesis

Simple esters

As mentioned in the experimental section, the transesterification reaction of NPAME to NPAIE was attempted under a variety of conditions (table 8). After establishing the quantities of carboxyl esterase and NPAME desired for the transesterification reaction, studies were undertaken to determine if the reaction rate could be increased by changing the isopropanol concentration and percent water content of the reaction mixture.

Figure 8 shows the relationship between the reaction rate (units/gram) and isopropanol concentration (M). The reaction mixtures in table 8 containing 22 mg NPAME and 2.0 mg

carboxyl esterase in 5.0 ml solutions containing 0.1% water were monitored by HPLC for isopropyl ester formation using a Baker C₁₈ reverse phase column with an eluent of 0.1M potassium phosphate pH 7.0 containing 30% acetonitrile. Figure 8 shows that for these particular conditions a 1.5M isopropanol concentration gives the optimum reaction rate.

The reaction mixtures in table 9 containing 22 mg of NPAME, 0.6M isopropanol, and 2.01 mg carboxyl esterase and various percentages of water were monitored for isopropyl ester formation by the same HPLC method previously mentioned in this section. Figure 9 shows the relationship between the reaction rate and percentage water in the reaction mixture. From this figure, it is evident that the reaction with 0.15% water gives the fastest rate under these particular conditions.

The beta-nitropropionic acid methyl ester was reacted with 1-butanol in the presence of lipase or carboxyl esterase (conditions mentioned in the experimental section). The reaction with the lipase showed no signs of butyl ester production. However, the reaction with carboxyl esterase did show evidence of butyl ester formation. A control for this transesterification reaction (containing no enzyme) showed no evidence of butyl ester formation.

These reactions were monitored using the same HPLC technique mentioned previously in this section. Figure 10 shows the chromatogram of the carboxyl esterase transesterification of the nitropropionic acid methyl ester with 1-butanol just as solutions were mixed and figure 11 shows this same reaction 20 hours later.

Figure 11 shows the same two peaks seen in figure 10 (a NPA peak followed by a larger NPAME peak), but an additional peak is present in figure 11 following these two peaks. This peak elutes off the column at the same location as the isopropyl ester and is probably the butyl ester. The appearance and location of the third peak indicates that transesterification has taken place. During this reaction it was observed that an aqueous layer (5% v/v) developed which was probably due to the lyophilized enzyme. Apparently small amounts of water may optimize reaction conditions -- as seen with the transesterification reaction to -- prepare the isopropyl ester mentioned earlier.

Polysaccharide esters

As mentioned in the experimental section, synthesis of the nitropropionic acid derivative of cellulose by transesterification was attempted by first absorbing either lipase or carboxyl esterase onto the cellulose then rotating the mixture with NPAME. To determine whether enzymatic synthesis occurred, the cellulose samples were treated with a solution of HCl, and the resulting solution was then analyzed for nitropropionic acid by HPLC chromatography (experimental

section). The presence of nitropropionic acid in the HCl solution would indicate synthesis of the desired product.

Figure 12 shows the chromatogram corresponding to the controls (chromatograms of all controls were identical). Each control resulted in a straight base line except for a small artifact located at 5 ml on the chromatogram. The artifact is probably caused by a difference in buffer concentration between the applied samples and the HPLC eluent. The chromatograms of all the other cellulose samples were also identical to figure 12 except for the cellulose sample soaked with lipase solution, 3.65 mg/ml.

Figure 13 shows the chromatogram of the cellulose sample prepared by soaking with a lipase solution (experimental section). In figure 13 a peak that is larger than the artifact in figure 12 is present. This peak elutes at void volume (5 ml) of column which is where nitropropionic acid also elutes. It appears that the transesterification synthesis of the cellulose derivative of nitropropionic acid is possible using lipase as a catalyst.

Organic synthesis

The transesterification reaction of the NPAME with beta-methyl-D-glucoside was studied in the presence and absence of catalyst (see experimental section). The desired product is the tetra NPA derivative of beta-methyl-D-glucoside. Analysis of the reaction mixture was accomplished with the same chromatographic technique used to analyze the degree of esterification of beta-methyl-D-glucoside.

NPAME has an elution volume of approximately 136 ml (figure 14) while the desired product elutes at approximately 48 ml (figures 6 and 7). Application of the product of the transesterification reaction in the presence of catalyst indicates that most of the starting ester is unreacted (figure 15). Greater conversion is accomplished in the absence of catalyst as indicated in figure 16. Unfortunately the conversion is still incomplete, so organic transesterification does not appear to be the desirable route to prepare the tetra B-NPA derivative of beta-methyl-D-glucoside.

MATERIALS

A list of the enzymes used in this project with the catalog and lot numbers are given below. Unless otherwise indicated the supplier was Sigma Chemical Company:

- 1) No. A-9128 D-amino acid oxidase, Lot # 14 F
 - 2) No. B-2252 bromelain, Lot # 113-F-0585
 - 3) No. E-3128 carboxyl esterase, Lot # 34-F-8110
 - 4) No. C-2770 cholesterol esterase, Lot 109-C-40211
 - 5) No. C-4129 alpha-chymotrypsin, Lot # 109-C-8045
 - 6) No. F-8629 ficin, Lot # 13-F-0808
 - 7) No. L-1754 lipase, Lot # 34-F-0621
 - 8) No. P-3250 papain, Lot # 93-F-0182
 - 9) No. P-7125 pepsin, Lot # 63-F-0538
 - 10) Code HRP-3C peroxidase batch 697 AX from Accurate Chemical & Scientific Corp.
 - 11) Code RN-1 ribonuclease batch 50-AX from Accurate Chemical & Scientific Corp.
- Additional reagents were obtained from the following sources:

- 1) American Scientific Products
- 2) Aldrich Chemical Co.
- 3) Pierce Chemical Co.
- 4) Sigma Chemical Co.
- 5) Mallinckrodt, Inc.

Whenever available, the chemicals purchased were of reagent grade quality.

Column packing materials for liquid chromatography were purchased from the following companies:

- 1) Bio-Rad Laboratories
- 2) E.M. Science
- 3) J.T. Baker Chemical Co.
- 4) Pharmacia, Inc.

EXPERIMENTAL

Oxidation of beta-alanine

Chemical oxidation

To synthesize NPA, the direct chemical oxidation of beta-alanine (0.5M) with hydrogen peroxide (6%) was attempted in a solution containing 0.08M NaOH. After mixing for 60 hours at room temperature, concentrated HCl was added to the solution to adjust the pH to 1.5. The solution was extracted with ethanol, and the ethanol extract was dried over CaCl_2 and evaporated to produce a yellow oil. Excess hydrogen peroxide was destroyed by incubating with peroxidase.

Enzymatic oxidation

Reaction mixtures containing 0.1M beta-alanine, 0.1M buffer (tris acetate and potassium phosphate pH 7 to 8), 0.037% hydrogen peroxide and 0.02% peroxidase were used to investigate the enzyme catalyzed oxidation of beta-alanine. Two controls were prepared identical to the above reaction except one contained no hydrogen peroxide, and the other contained no peroxidase. All of the reactions were incubated in the dark at both 23°C and 37°C. The oxidation of beta-alanine by hydrogen peroxide can be monitored spectrophotometrically. NPA has an absorption spectrum in the ultraviolet region with a peak at approximately 265 nm. The absorptivity at this peak is measured in our laboratory to be $23.7 \text{ M}^{-1} \text{ cm}^{-1}$.

Nitration

Oxidation of nitroalkanes with either glucose oxidase or D-amino acid oxidase produces H_2O_2 . A system consisting of a small 1 ml column of glass beads, pump, and electrochemical sensor was set up. A 0.2M phosphate buffer, pH 7.2, containing 0.1M KCl and 50 PPB of bioban was constantly pumped through the column. After passing through the column, the solution passes over an electrochemical sensor, so these oxidation reactions can be monitored by injecting reaction solutions into the system and measuring the H_2O_2 produced (40).

Glucose oxidase was checked for its ability to oxidize nitroethane, nitropropane and nitropropionic acid. In one reaction, glucose oxidase was incubated with a 12 mM nitroethane solution in a 0.2M acetate buffer, pH 5.6, containing 0.1M KCl, 0.05% tween and 50 PPB bioban. In another reaction, the glucose oxidase was incubated with a 50 mM nitropropane solution in a 0.2M phosphate buffer, pH 7.2, containing 0.1M KCl, 0.05% tween 20 and 50 PPB bioban.

A series of reactions was performed to measure the oxidation of nitropropionic acid by glucose oxidase. All reactions were carried out in a 0.2M phosphate buffer containing 0.1M KCl, 0.05%

tween and 50 PPB bioban. There were six reactions of nitropropionic acid and glucose oxidase, and the following is a list of the different NPA concentrations and pH values of buffer used for these reactions:

- 1) 0.03M, pH 6.8
- 2) 0.05M, pH 5.6
- 3) 0.06M, pH 4.0
- 4) 0.05M, pH 3.8
- 5) 0.2M, pH 3.0
- 6) 0.16M, pH 2.8

D-amino acid oxidase was mixed with both nitroethane and NPA at pH 7.0, 8.0 and 8.3. In a separate experiment, it was discovered that the solution of D-amino acid oxidase causes rapid disappearance of a standard H_2O_2 solution. The breakdown of H_2O_2 could be caused by the presence of catalase as an impurity in the D-amino acid oxidase obtained from Sigma Chemical Company.

Because oxidation of nitroalkanes with horse radish peroxidase generates carboxyl compounds and HNO_2 , it was necessary to use a method capable of monitoring the nitrite produced. An Orion nitrite electrode was suitable for this purpose. A reaction mixture containing horse radish peroxidase (19 mg/ml), 0.14M nitropropane, and 0.002M phosphate, pH 7.3, was monitored with a nitrite electrode.

The reverse of the reaction or nitropropane synthesis using propionaldehyde and $NaNO_2$ in the presence of horse radish peroxidase was then attempted. Twelve mg propionaldehyde and 0.014 g $NaNO_2$ were mixed with 0.5 ml of a horse radish peroxidase solution (0.05 mg/ml) prepared in a 0.02M phosphate buffer. This reaction was performed at pH values of 5.8, 6.2, 6.9, and 7.8 and monitored by measuring the decrease in nitrite with a nitrite electrode.

Preparation of semisynthetic enzymes

A technique for preparing semisynthetic enzymes was discussed. The general procedure for this synthesis is as follows:

- 1) The enzyme to be modified is dissolved in deionized water at the desired concentration, and HCl is used to titrate this solution to an acid pH (3). This solution is mixed at room temperature for one hour.
- 2) A modifier is then added at the desired concentration to inhibit the enzyme, and mixing of the solution is continued for another three hours at room temperature.
- 3) Using NaOH, the solution is then titrated to an alkaline pH (8-9). After cooling this solution to 10°C, a crosslinker is added at the desired

concentration. This solution continues to be mixed for 16 more hours at 10°C.

- 4) The enzyme is now modified, but before analysis it must be further purified by dialysis for 48 hours against either deionized water or low concentration (2-5 mM) acetate buffer (pH 6-7.5).

If during any of these procedures the pH of the solution changed after adding either a modifier or a crosslinker, the pH was titrated back to where it was originally using either HCl to lower it or NaOH to raise it. Table 6 lists the desired concentrations and various combinations of enzymes, modifiers, and crosslinkers used to prepare the modified enzymes.

Hydrolysis

Non-enzymatic

Table 3 lists the types and concentrations of solutions used, and also the pH values of these solutions after adding ENA. The ENA was allowed to incubate for 20 hours at room temperature. The ethanol released was measured using Sigma's diagnostic kit # 332-UV which is a spectral, enzymatic kit containing alcohol dehydrogenase and NAD. At a wavelength of 340 nm, NAD has low absorptivity while NADH has a high absorbitivity, so ethanol production can be monitored at this wavelength. A standard plot of absorbance (340 nm) versus micromoles of ethanol is prepared for comparison with the hydrolyzed ENA solutions.

Enzymes

The reaction of ester hydrolysis to acid and alcohol in the presence of a natural enzyme was monitored by two different methods. One method was using Sigma's ethanol determination kit described in the previous section, but instead of incubating the esters for 20 hours before checking the solution once for ethanol, these solutions were checked for ethanol several times within the first hour of incubation. Thus, one can obtain the hydrolysis rate in micromoles of ethanol produced per minute and these values determine the activity (table 4).

The other method is an HPLC (high performance liquid chromatography) procedure. Esters can be separated from their corresponding acid using a reverse phase C₁₈ liquid chromatography column with an eluent of potassium phosphate pH 7.5, containing 33% acetonitrile. By injecting the ester incubation solution into the HPLC system several times within one hour, we were able to monitor both ester reacted or acid produced to obtain micromoles/min (unit) value necessary for calculating the activity measurement in table 2. In this manner, the rate of hydrolysis in a sample containing enzyme and in the control was determined.

Esterification

Enzymatic synthesis of esters

Mixtures of 0.25M NPA and 10% lipase (Candida cylindracea) were prepared in both isopropanol and 1-butanol. These materials were mixed by automatic rotation at a rate of 15 rpm. Five hours after mixing the materials these mixtures were filtered (0.2 μ m) to remove the lipase.

Using an eluent containing 33% acetonitrile in (0.01M) potassium phosphate, pH 7.3, NPA can be separated from its corresponding esters by HPLC. The alcohol removed from each sample was diluted 1:10 with the HPLC eluent before injecting it into the system.

Esterification was also attempted using NPA and methanol in the presence of carboxyl esterase to prepare NPAME. Carboxyl esterase was immobilized for one set of experiments and absorbed for another set. Carboxyl esterase was entrapped onto Sepharose (R) 2B (Pharmacia) gel (23). 0.75 grams of this gel containing 0.17 mg of carboxyl esterase were mixed into the following solutions which contained potassium phosphate pH 7.0 buffer (0.1M before being added to methanol) and 0.5 mg/ml NPA:

- 1) 20% methanol, 80% buffer
- 2) 40% methanol, 60% buffer
- 3) 60% methanol, 40% buffer
- 4) 10% methanol, 45% buffer and 45% glycerine.

The carboxyl esterase was removed, after mixing these solutions on an automatic rotator at 100 rpm for fifteen hours at room temperature. It was noted that a reverse phase C₁₈ (Baker) HPLC column could be used to separate esters from their corresponding acids using 10% acetonitrile in potassium phosphate pH 7.0 as eluent. Each of the four solutions were analyzed using this HPLC technique.

The carboxyl esterase was immobilized onto Sepharose (R) 6B-CL gel (Pharmacia) (41). The gel (0.3 g) contained 0.18 mg of carboxyl esterase and was mixed with the following solutions which all contained 0.2 mg/ml NPA and potassium phosphate buffer, pH 7.0, (0.1M before being added to methanol):

- 1) 70% methanol, 30% buffer
- 2) 45% methanol, 10% buffer, 45% glycerol
- 3) 20% methanol, 20% buffer, 30% glycerol, 30% ethylene glycol
- 4) 40% methanol, 20% buffer, 40% ethylene glycol

The immobilized carboxyl esterase was removed and these solutions were analyzed by the same HPLC method used to analyze the absorbed carboxyl esterase reaction solutions.

Organic synthesis of esters

Simple esters. The methyl, ethyl, isopropyl, and isobutyl esters of nitropropionic acid were synthesized by organic means in our laboratory. Different techniques were investigated for these syntheses until a preferred route was ascertained.

The methyl ester was first synthesized by esterification of NPA in absolute methanol using trace amounts of concentrated sulfuric acid (42). The ester product was isolated by vacuum distillation.

The ethyl ester was first synthesized by metathesis using silver nitrite and ethyl-3-bromopropionate (5). Hexane was chosen as the solvent, and vacuum distillation was necessary to isolate the ester.

The methyl and ethyl esters were then both prepared by direct esterification by refluxing NPA with an excess of the corresponding alcohol (methanol or ethanol) in the presence of a strongly acidic (sulfonic) ion exchange resin (Dowex^(R) 50 X 8-100 from Aldrich). 2,2-dimethoxy propane was added to the methyl ester synthesis and 2,2-diethoxy propane was added to the ethyl ester synthesis to favor equilibrium conditions by scavenging the elements of water produced by the reactions. These ketals react with water to release the corresponding alcohol and acetone. The esters were isolated by filtration and evaporation of the volatile components (alcohol, excess ketal and acetone) with the aid of a rotary evaporator at low temperature.

The optimum synthetic route is similar to the procedure just mentioned, except a trap consisting of size 3A molecular sieves was used to remove water produced during the reaction. The presence of the molecular sieve eliminates the need for adding a ketal to the reaction mixture to remove the water produced. The ester is now isolated by removing the alcohol on a rotary evaporator at room temperature. The methyl, isopropyl, and isobutyl esters of NPA were all prepared in this manner.

Monosaccharide esters. The liquid chromatography method used for analysis of monosaccharides is described in the section "Organic synthesis of esters-monosaccharide esters" on page 11. Different methods of direct esterification were investigated to synthesize the tetra NPA derivative of either alpha- or beta-methyl-D-glucoside.

By the initial method, beta-methyl-glucoside was dissolved in hot 1,4 dioxane along with NPA. Four and one-half moles of NPA were used for every one mole of beta-methyl-D-glucoside (to ensure tetra substitution). The reaction mixture was refluxed using a trap containing size 5A molecular sieves to remove water produced during the reaction. The excess dioxane was removed using a rotary evaporator and the final product was

analyzed by liquid chromatography.

A synthesis route identical to the initial method was studied with the exception that a strongly acidic (sulfonic) ion exchange resin was added as a catalyst. The product was isolated by first filtering out the catalyst, then using a rotary evaporator to remove the dioxane. Liquid chromatography was used to analyze the product (section entitled "Organic synthesis of esters - Monosaccharide esters").

In the final synthesis procedure, NPA was melted in a hot water bath. Alpha-methyl-D-glucoside (0.23 moles) was added for every mole of melted acid to ensure a tetra substituted derivative. When the entire mixture was melted, it was removed from the hot water bath and allowed to solidify at room temperature. The solid mixture was then heated to 85°C under vacuum (mechanical pump) for 75 hours. The product was then analyzed by liquid chromatography.

Transesterification

Enzymatic transesterification

Both 1-butanol and isopropanol were reacted with NPAME (section entitled "Transesterification - Enzymatic synthesis - Simple esters") in the presence of an enzyme to study transesterification.

NPAME dissolved in isopropanol was mixed with carboxyl esterase. The carboxyl esterase was dialyzed against 0.002M phosphate buffer (varying the pH for different reactions). These solutions were then lyophilized to produce an enzyme, buffered salt mixture of a desired pH. The mixture and reactants were added to hexane. These reactions were carried out at different temperatures while varying the amounts and concentrations of reactants along with the enzyme, pH and percent water in the reaction mixture. Table 8 lists these conditions. Each individual reaction mixture was shaken for 20 hours. A control was set up for each of the reactions by mixing the same solutions shown in table 8 except leaving out the carboxyl esterase, and adding only lyophilized phosphate buffer.

In another series of transesterification experiments, 1,2 dichloroethane was selected as the solvent in place of hexane. The reaction mixture consisted of 5.0 ml of 1,2 dichloroethane containing the following:

- 1) 8.8 mg NPAME
- 2) 0.3M isopropanol
- 3) 1.9 mg carboxyl esterase

No water was added to the reaction which was carried out at room temperature. The reaction mixture was analyzed by HPLC in the same manner as the reactions using hexane as solvent.

The organic solvents were removed from the reacted solutions by evaporation, and the residue was analyzed using HPLC with a C₁₈ reverse phase column and an eluent consisting of 30% acetonitrile in 1.1M potassium phosphate buffer, pH 7.0. There was no evidence of NPAIE synthesis by transesterification.

Transesterification reactions were also investigated with the NPAME in 1-butanol in the presence of both lipase and carboxyl esterase. Neither additional organic solvents nor water were added.

In one test tube 5.0 ml of 1-butanol, 1.2 mg of carboxyl esterase (lyophilized in potassium phosphate), and 63 mg of NPAME were mixed. Another test tube contained the same mixture except that 0.5 grams of lipase were used in place of the lyophilized carboxyl esterase. A control was also included which contained 5.0 ml of 1-butanol and 63 mg of NPAME. All three test tubes were shaken for 20 hours at room temperature. The products from these reactions were analyzed by HPLC using a C₁₈ reverse phase column with 0.01M potassium phosphate, pH 7.3, containing 33% acetonitrile as an eluent (section entitled "Transesterification - Enzymatic synthesis - Simple esters").

Polysaccharide esters

Enzymatic means were employed to attempt the transesterification synthesis of the NPA derivative of cellulose.

In the first series of experiments lipase and carboxyl esterase were investigated. In one test tube, 1.0 gram of cellulose was added to a test tube containing 4.0 ml of a 2.2 mg/ml lipase solution prepared in 0.005M potassium phosphate, pH 7.3. The slurry was periodically mixed with a metal spatula for 1.5 hours to absorb enzyme onto the cellulose. Liquid was then removed by decanting after centrifuging.

Three ml of nitropropionic acid methyl ester (experimental section entitled "Esterification - Organic synthesis of esters - Simple esters") were added to the lipase saturated cellulose. In another test tube, the 2.2 mg/ml lipase solution was replaced with a 2.0 mg/ml carboxyl esterase solution. A control was included for both reactions by replacing the enzyme solution with the 0.005M potassium phosphate, pH 7.3, used to prepare the enzyme solutions. The test tubes were wrapped in aluminum foil and allowed to mix at room temperature for 6 days at 25 rpm.

After the enzyme treated cellulose samples reacted with the ester for 6 days, the contents of the test tubes were centrifuged and the ester was discarded. The cellulose compounds were then washed with hexane and water. One ml of 0.2N HCl was added, to each of the three test tubes containing cellulose samples, and allowed to saturate the samples for 1.5 hours. A sample (0.35 ml) of each HCl solution was added to 0.065 ml of 2N NaOH, and 0.35 ml of HPLC eluent. A reverse phase C₁₈ column

(Baker) was used with an eluent of 0.1M potassium phosphate, pH 7.3, containing 33% acetonitrile. The sample solution was filtered (0.2 μ m) and then passed through the HPLC column.

In the second series of reactions for the transesterification synthesis of cellulose derivatives, only lipase was tested for catalytic activity. This synthesis reaction was performed identically to the cellulose transesterification reactions mentioned earlier except the following amounts of reactants were employed: 1) 5.0 ml of a 3.65 mg/ml lipase solution (prepared in 0.005M potassium phosphate, pH 7.3) 2) 0.3 g cellulose and 3) 2.0 ml of nitropropionic acid methyl ester. A control was also included with the same quantities of materials but the 0.005M potassium phosphate, pH 7.3, solution used to prepare the enzyme solution was replaced for the lipase solution.

These products were incubated with the HCl solution, and the HCl solution was then analyzed for NPA groups using the same HPLC eluent and column as used previously to analyze the cellulose samples. Six-tenths ml of 0.2N HCl was mixed with the cellulose derivative and its control for 24 hours. This HCl solution (0.35 ml) was added to 0.35 ml of HPLC eluent and 0.65 ml of 2.0M NaOH. The resulting mixture was filtered (0.2 micron), then passed through an HPLC column. These results have been discussed in the section entitled "Transesterification - Enzymatic synthesis - Polysaccharide esters".

Organic transesterification of esters

The tetra NPA derivative of beta-methyl-D-glucoside was synthesized by transesterification. Two reactions were performed. In both reactions, beta methyl-D-glucoside and NPAME (synthesis mentioned earlier in this experimental section) were dissolved in hot 1,4-dioxane. Four and one-half moles of the ester were used for every mole of beta-methyl-D-glucoside. A highly acidic (sulfonic) ion exchange resin (Dowex[®]) was added in one reaction. Both reaction mixtures were refluxed while water and methanol were removed from the reaction using a trap containing size 5A molecular sieves. The catalyst was removed from the one reaction by filtration, and dioxane was removed from both solutions using a rotary evaporator. As mentioned previously in this report both products were analyzed using liquid chromatography (section entitled "Transesterification - Organic synthesis").

DELIVERABLE

One thousand grams of the tetra NPA derivative of alpha-methyl-D-glucoside were delivered to the contractor in two shipments. The first shipment of 850 grams was sent on October 15, 1986 and the remainder of the required deliverable was sent on October 31, 1986. The procedure for synthesis of this derivative is described in the experimental section entitled "Esterification - Organic synthesis of esters - Monosaccharide esters".

The deliverable was analyzed by gel permeation chromatography which indicated that the mole percent ester is approximately 85. Elemental analysis was not performed since the composition for the starting mixture is almost the same as the product.

CONCLUSIONS

The objective of this program was the enzymatic esterification of NPA to cellulose. A further objective was the enzymatic synthesis of NPA. To achieve these objectives, both natural and semisynthetic enzymes were employed. The achievements under the contract are:

1. Analysis of the transesterification reaction of cellulose with the NPAME in the presence of lipase indicated that the reaction was indeed catalyzed by the enzyme (see section "Polysaccharide esters", page 12).

2. Enzymatic catalysis of the nitration of propionaldehyde (see section "Nitration", page 7).

3. A deliverable consisting of NPA ester of alpha-methyl-D-glucoside was prepared by organic synthesis techniques (see section "Monosaccharide esters", page 11).

Enzymatic catalysis of the transesterification reaction would fulfill one objective of the contract. When lipase was used as a catalyst, the evidence indicates that some NPA ester of cellulose did indeed form. The concentration of the ester was so low that extraction of this product was not practical.

Enzymatic catalysis of the nitration reaction is a key reaction in the synthesis of NPA. Evidence that peroxidase catalyzes this reaction has also been presented.

RECOMMENDATIONS

Almost all of the conclusions discussed above are preliminary and require further research to confirm. Two results show promise for developing enzymatic methods for the nitration of cellulose:

1. Enzymatic catalysis of the nitration of propionaldehyde (see section "Nitration" page 7).

2. The transesterification reaction of cellulose with the methyl ester of NPA which was apparently catalyzed by lipase (see section "Polysaccharide esters" page 12).

Since horseradish peroxidase can catalyze the nitration of propionaldehyde, it may be possible to use this enzyme for the nitration of cellulose by the following scheme. First the hydroxyl groups of the cellulose are oxidized to aldehyde groups either by enzymatic catalysis or classical chemical means. Then horseradish peroxidase is used to catalyze the reaction between the cellulose derivative containing aldehyde groups and the nitrite ion. The product of this reaction is nitrocellulose, an excellent and widely used gun propellant. It is highly recommended that this enzymatic method of preparation of nitrocellulose be explored and developed. The enzymatic method would not be nearly as energy intensive or require the high pressure of the classical chemical methodologies. Some effort could also be expended on the transesterification reaction listed in 2 above although it is believed to be of less importance. The product of the transesterification reaction is a NPA ester of cellulose rather than nitrocellulose.

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GLOSSARY

BSA:	bovine serum albumen
DA:	dimethyl adipimidate
DP:	dimethyl pimilimidate
DS:	dimethyl subermidate
EB:	ethyl butyrate
ENA:	ethyl nitroacetate
G:	glutaraldehyde
IPA:	indole propionic acid
NE:	nitroethane
NM:	nitromethane
NP:	nitropropane
NPA:	beta-nitropropionic acid
NPAEE:	beta-nitropropionic acid ethyl ester
NPAME:	beta-nitropropionic acid methyl ester
NPAIE:	beta-nitropropionic acid isopropyl ester
PPB:	parts per billion
RNase:	ribonuclease
TrEE:	tryptophan ethyl ester

Table 1. Enzymatic oxidation of beta-alanine

Sample #	Peroxidase (mg)	Temperature °C	Incubation time (hr)	pH	Control total abs (265nm)	Sample abs (265nm)
1	0.4	23	0.5	7.0	0.15	0.145
1	0.4	37	2.0	7.0	0.15	0.15
2	0.4	23	0.5	7.5	0.15	0.145
2	0.4	37	2.0	7.5	0.145	0.14
3	0.4	23	0.5	8.0	0.17	0.14
3	0.4	37	2.0	8.0	0.15	0.13
4	0.2	23	0.5	7.4	0.13	0.125
4	0.2	23	1.0	7.4	0.13	0.12
4	0.2	23	4.3	7.4	0.11	0.115
4	0.2	23	21.0	7.4	0.15	0.14
5	0.4	23	0.5	7.4	0.145	0.14
5	0.4	23	1.0	7.4	0.15	0.12
5	0.4	23	4.3	7.4	0.15	0.14
5	0.4	23	21.0	7.4	0.17	0.17

Table 2. Enzymatic oxidation of nitro-aliphatic compounds

Substrate	Substrate conc. (M)	pH	Activity (U/G)
Nitroethane	0.012	5.6	23 \pm 4.3
Nitropropane	0.050	7.2	6 \pm 1.2
Nitropropionic acid	0.03	6.8	0
" "	0.05	5.6	"
" "	0.06	4.0	"
" "	0.05	3.8	"
" "	0.2	3.0	"
" "	0.16	2.8	1.7 \pm .65

Table 3. Specific activity of natural enzymes toward nitro esters

Enzyme	Substrate	Hydrolysis pH	Analysis method	Specific activity (U/G)
Bromelain	NPAME	6.3	HPLC	0
Bovine serum Albumen	"	7.5	"	0
Carboxyl Esterase	ENA	8.5	Spectral	1370
"	"	8.0	"	1400
"	"	7.5	"	1840
"	"	7.0	"	1980
"	"	"	HPLC	829
"	"	6.8	Spectral	2000
"	"	"	"	2000
"	Ethyl butyrate	7.2	"	197,000
"	"	"	"	200,000
"	NPAAE	6.5	HPLC	10,000
"	NPAIE	6.9	"	9000
"	NPAME	7.0	"	4100
Cholesterol Esterase	ENA	6.1	Spectral	2.6 \pm 1.3
"	"	6.7	"	0.92 \pm 0.25
"	NPAME	7.1	"	0
"	Ethyl butyrate	7.0	"	28.2 \pm 0.52
Chymotrypsin	NPAME	7.5	HPLC	1
"	ENA	5.7	Spectral	3.2 \pm .4
Ficin	NPAME	7.5	HPLC	0
Hemoglobin	"	6.5	"	0
Lipase	"	7.0	"	1.5 \pm .05
"	NPAEE	7.3	Spectral	7.8 \pm .98
Papain	ENA	7.4	"	0
"	NPAME	6.5	HPLC	0
Pepsin	ENA	7.4	Spectral	0
"	NPAME	3.0	HPLC	0
Protease	"	6.5	"	0
Ribonuclease	ENA	7.2	Spectral	0
Trypsin	NPAME	7.5	HPLC	0

Table 4. Variation in specific activity of carboxyl esterase towards the hydrolysis of beta-nitropropionic acid ethyl ester

Carboxyl esterase concentration (mg/ml)	Activity (U/mg)	Monitored substance
2.0×10^{-3}	154.0	NPA
3.9×10^{-3}	82.9	"
5.7×10^{-3}	51.0	"
11.0×10^{-3}	4.9	"
2.0×10^{-3}	26.0	NPAEE
3.9×10^{-3}	60.0	"
5.7×10^{-3}	15.6	"
11.0×10^{-3}	2.1	"
9.9×10^{-4}	35.7*	"
9.9×10^{-4}	41.1*	NPA

* NPAEE purified by chromatography

Table 5. Specific activities of semisynthetic enzymes towards nitro esters

Starting enzyme	Modifier	Cross-linker	Substrate (pH)	Activity \pm error (U/G)
Ribonuclease	IPA1	G3	ENA ⁷ (7.5)	1.1 \pm 0.6
"	"	"	" (7.2)	1.0 \pm .16
"	"	"	TrEE ⁸ (7.5)	1.3 \pm .67
"	"	"	" "	1.1 \pm .15
"	"	"	EB ⁹ "	1.7 \pm .54
"	"	"	" "	1.9 \pm .79
"	"	DS ⁴	ENA (6.0)	1.0 \pm .4
"	NP ²	"	NPAEE ¹⁰ (7.4)	1.2 \pm .29
"	"	DP ⁵	" "	1.2 \pm .33
Ficin	"	"	ENA (7.6)	2.6 \pm .9
"	"	DS ⁶	NPAEE (8.0)	2.8 \pm 2.0
"	"	"	ENA (8.1)	4.1 \pm 3.6

1. IPA: Indole propionic acid
2. NP: Nitropropane
3. G: Glutaraldehyde
4. DS: Dimethyl subermidate
5. DP: Dimethyl pimilimidate
6. DS: Dimethyl subermidate
7. ENA: Ethyl nitroacetate
8. TrEE: Tryptophan ethyl ester
9. EB: Ethyl butyrate
10. NPAEE: Beta-nitropropionic acid ethyl ester

Table 6. Conditions for preparation of semisynthetic enzymes

Enzyme	Modifier	Cross-linker
0.6 mg/ml Bromelaine	5 mM NP	1.5 mM DS
"	"	1.5 mM DP
"	"	2.5 mM G
0.6 mg/ml BSA	"	"
"	"	1.5 mM DP
"	"	1.5 mM DS
0.6 mg/ml Chymotrypsin	"	"
"	"	1.5 mM DP
"	"	2.5 mM G
0.6 mg/ml Pepsin	"	"
"	"	1.5 mM DP
"	"	1.5 mM DS
0.6 mg/ml RNase	"	"
"	"	1.5 mM DP
"	"	2.5 mM G
"	5.4 mM NP	"
"	5.0 mM NE	"
"	"	1.5 mM DS
"	5.0 mM NM	"
"	"	2.5 mM G
"	1.0 mM IPA	"
"	"	1.8 mM DS
"	"	1.5 mM DS
"	NPA	1.7 mM DS
"	"	2.9 mM G
1.1 mg/ml Ficin	10 mM NP	2.9 mM DA
"	"	2.9 mM DP
1.0 mg/ml Ficin	5.0 mM NP	4.0 mM DS
"	"	"
"	5.0 mM IPA	"
"	"	7.3 mM DS
"	5.0 mM NPA	"
"	"	4.0 mM DS
0.88 mg/ml Ficin	"	8.8 mM DS
"	5.0 mM IPA	"
"	4.9 mM NP	"
0.6 mg/ml Ficin	6.0 mM NP	1.4 mM DS
"	"	1.3 mM DA
0.1 mg/ml Ficin	5.0 mM NP	1.5 mM DS
"	"	1.5 mM DP
"	"	2.5 mM G
0.09 mg/ml Ficin	4.9 mM NP	1.5 mM DS
"	"	2.8 mM DS
"	"	3.8 mM DS
"	5.1 mM NP	1.4 mM DS
0.034 mg/ml Ficin	3.7 mM NP	0.3 mM DP
0.03 mg/ml Ficin	5.0 mM NP	1.5 mM DS
"	"	1.5 mM DP
"	"	2.5 mM G

Table 7. Purity of aliphatic esters of beta-nitropropionic acid

Ester	Percent ester
NPA ethyl ester	89.5
NPA isobutyl ester	80.4
NPA isopropyl ester	83.0
NPA methyl ester	98.2

Table 8. Specific activity of carboxyl esterase towards the transesterification of beta-nitropropionic acid methyl ester with isopropanol

NPAME ¹ (mg)	Isopropanol (M)	C-esterase (mg)	pH	Temp. (°C)	% water	Activity (U/G)
8.8	0.3	1.9	7.0	23	0	0
"	"	2.2	7.5	"	"	"
9	"	1.16	"	40	"	"
9.04	"	"	"	18	"	"
55.6	1.5	1.87	6.8	"	"	"
100.8	3	6.4	"	"	"	"
55.6	1.5	1.52	"	"	"	"
12.4	0.3	1.66	"	"	"	"
50.8	.75	1.45	"	"	"	"
22	0.6	2.01	7.5	23	.03	1.14
"	"	"	"	"	.15	1.38
"	"	"	"	"	.3	0.74
"	"	"	"	"	.6	0.3
"	"	"	"	"	.9	0.3
"	.3	"	"	"	0.1	0.3
"	.5	"	"	"	"	0.6
"	.75	"	"	"	"	1.9
"	1.0	"	"	"	"	2.5
"	1.5	"	"	"	"	2.8
"	2.0	"	"	"	"	2.0
"	2.5	"	"	"	"	1.2
"	3.0	"	"	"	"	1.0
"	4.0	"	"	"	"	0.8
"	5.0	"	"	"	"	0.5

Each reaction mixture had a total volume of 5.0 ml.

1. NPAME is nitropropionic acid methyl ester

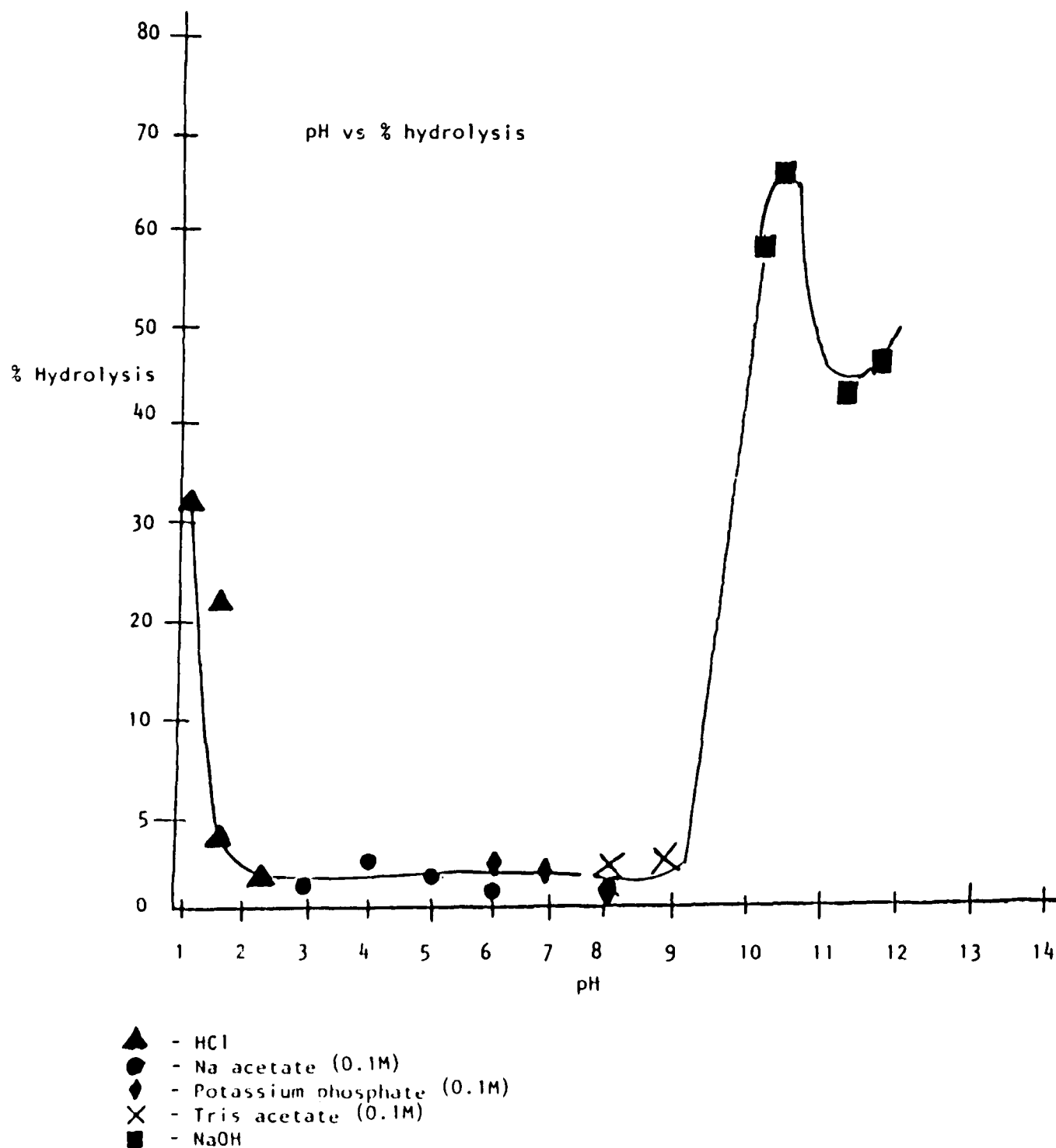


Figure 1. Ethyl nitroacetate hydrolysis as a function of pH

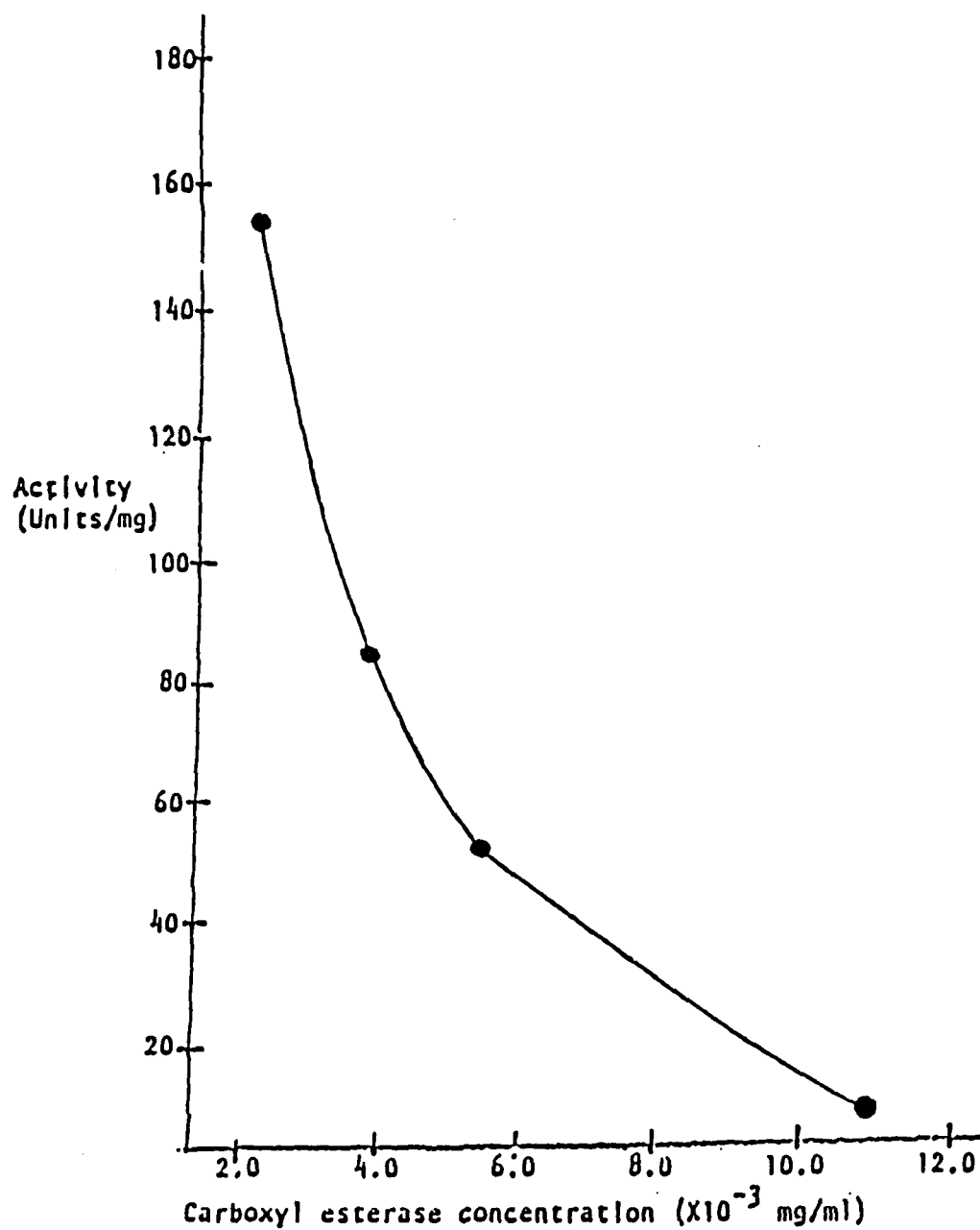


Figure 2. Specific activity of carboxyl esterase as a function of enzyme concentration

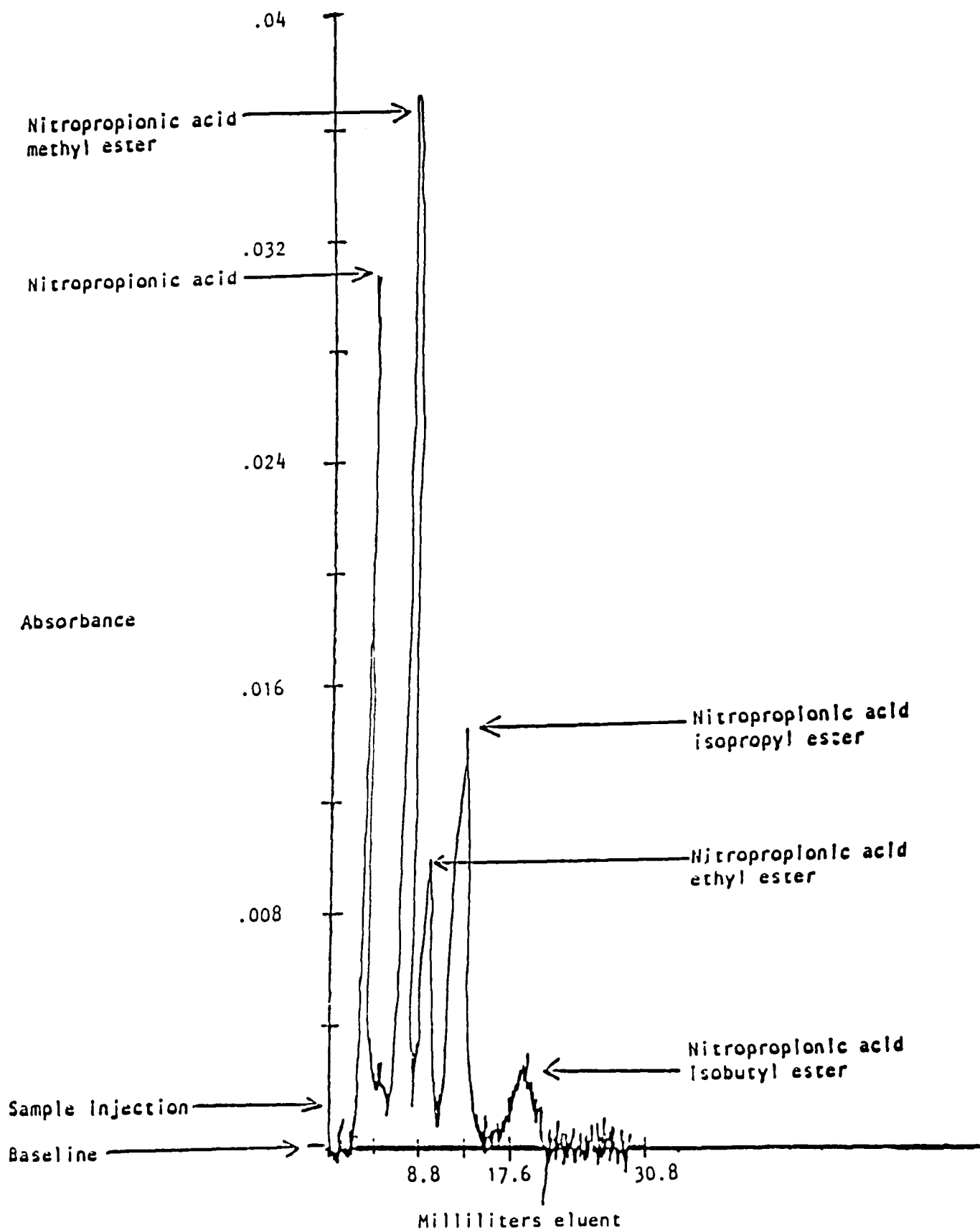


Figure 3. Chromatogram of the aliphatic esters of beta-nitropropionic acid using a C_{18} reverse phase support

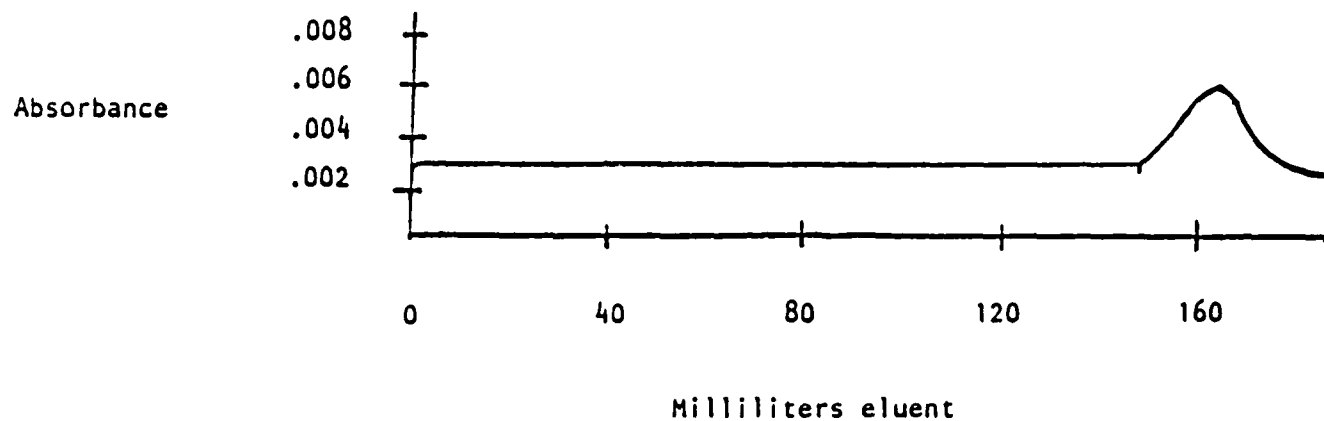


Figure 4. Chromatogram of beta-nitropropionic acid on a SephadexTM G-10 column

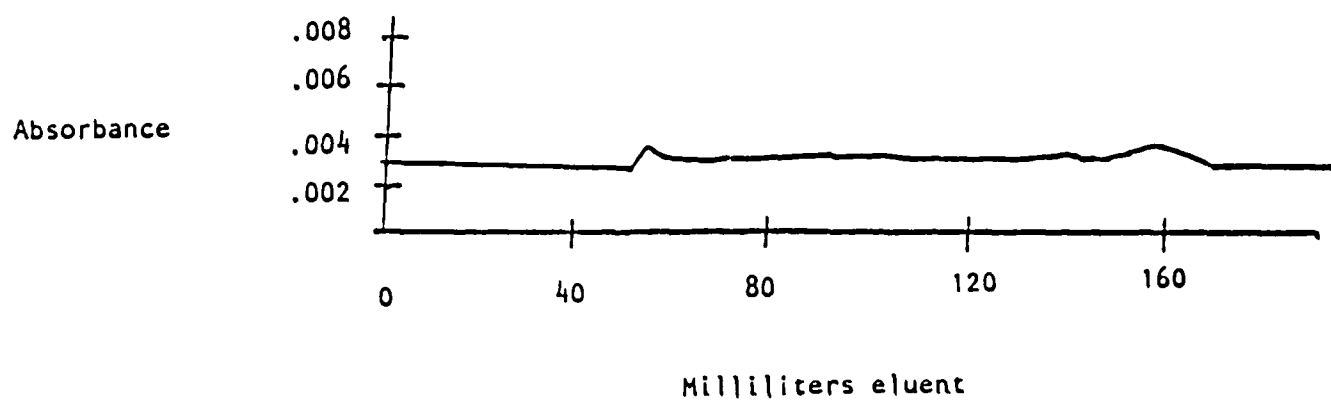


Figure 5. Chromatogram of the esters formed from the reaction of beta-nitropropionic acid and beta-methyl-D-glucoside in dioxane (SephadexTM G-10 column)

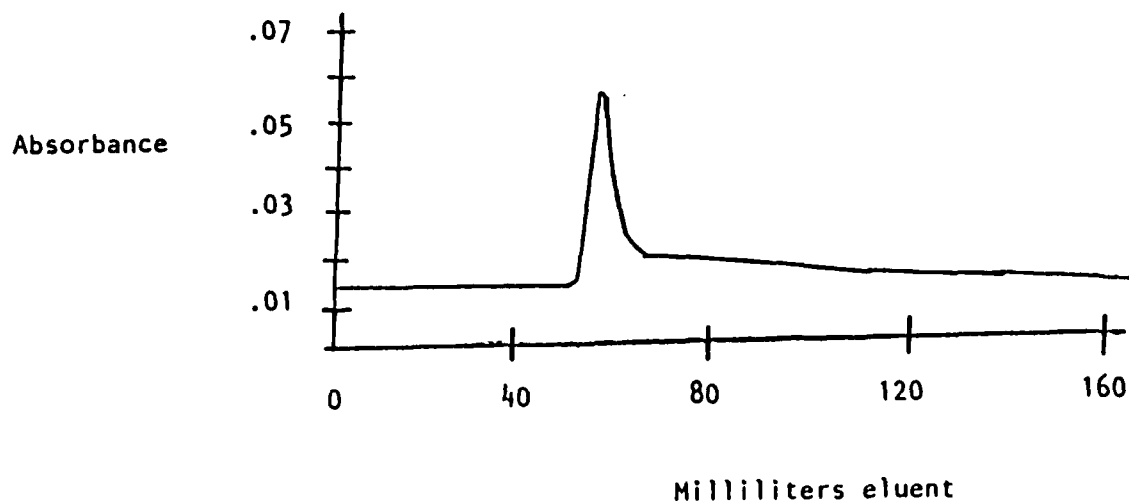


Figure 6. Chromatogram of the esters formed from the reaction of beta-nitropropionic acid and beta-methyl-D-glucoside in dioxane using a strong acidic ion exchange resin as a catalyst (SephadexTM G-10 column)

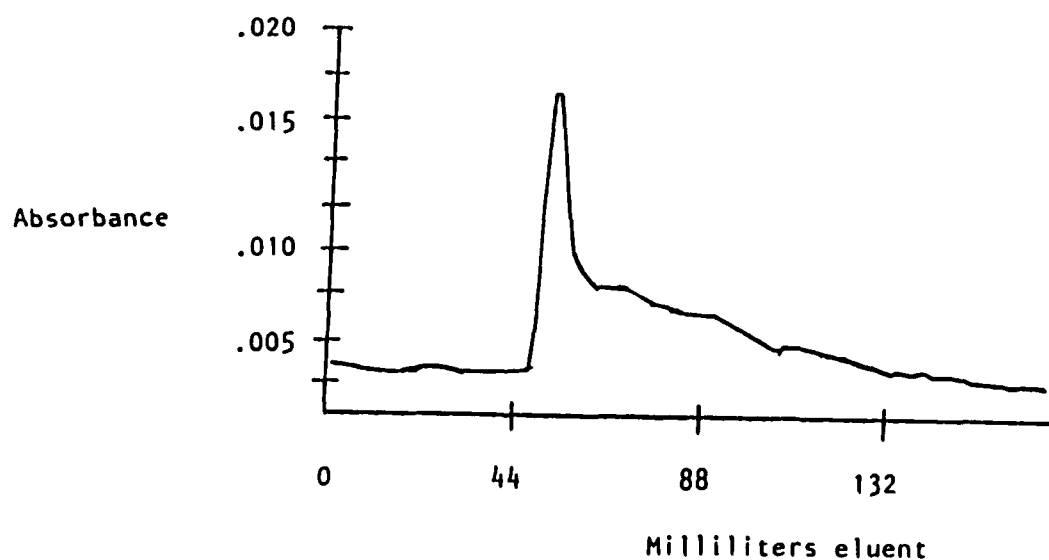


Figure 7. Chromatogram of the esters formed from the reaction of beta-nitropropionic acid and alpha methyl-D-glucoside in a melt (SephadexTM G-10 column)

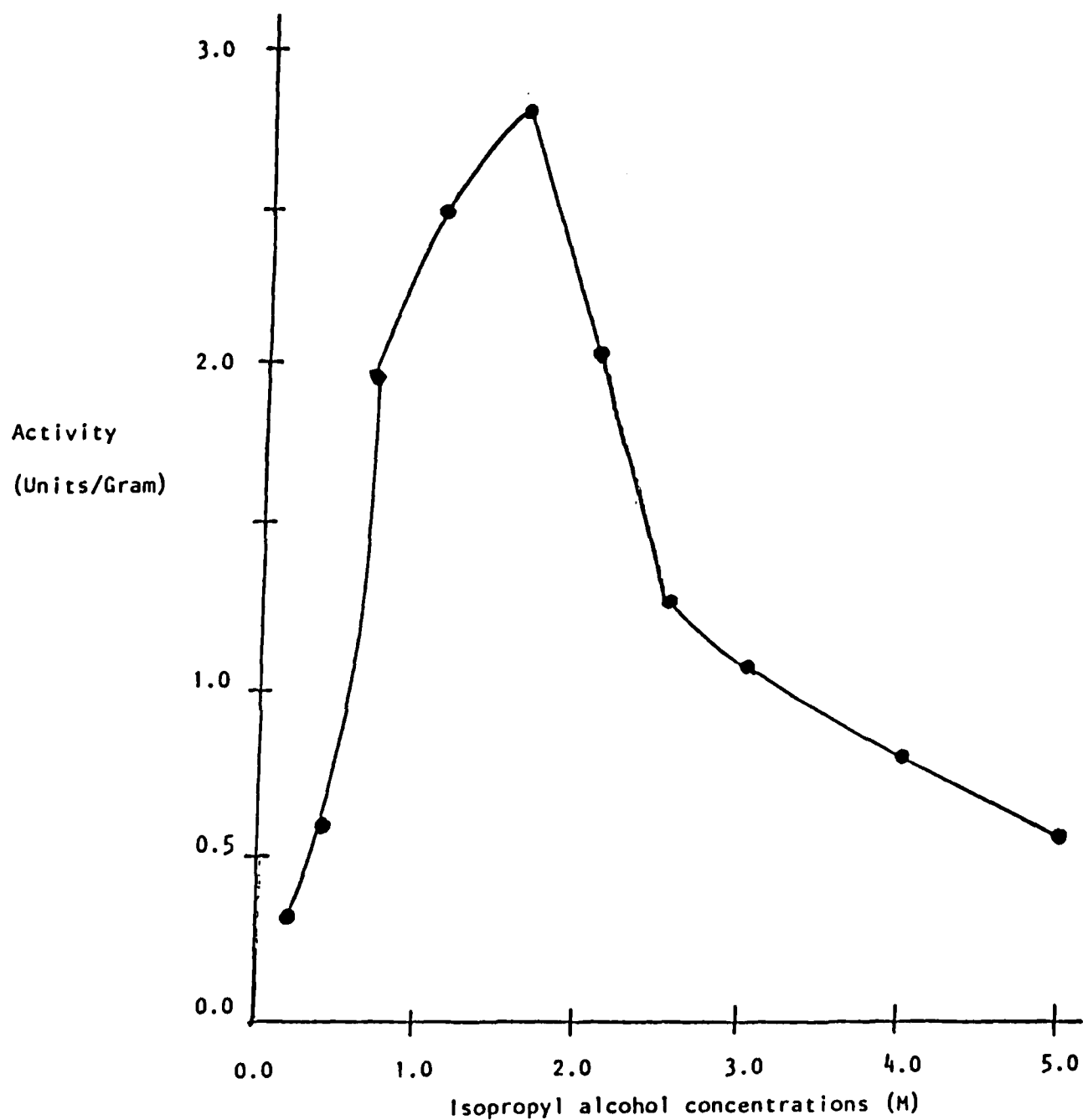


Figure 8. Activity of carboxyl esterase for the transesterification reaction between beta-nitropropionate methyl ester and isopropyl alcohol as a function of the initial isopropyl alcohol concentration

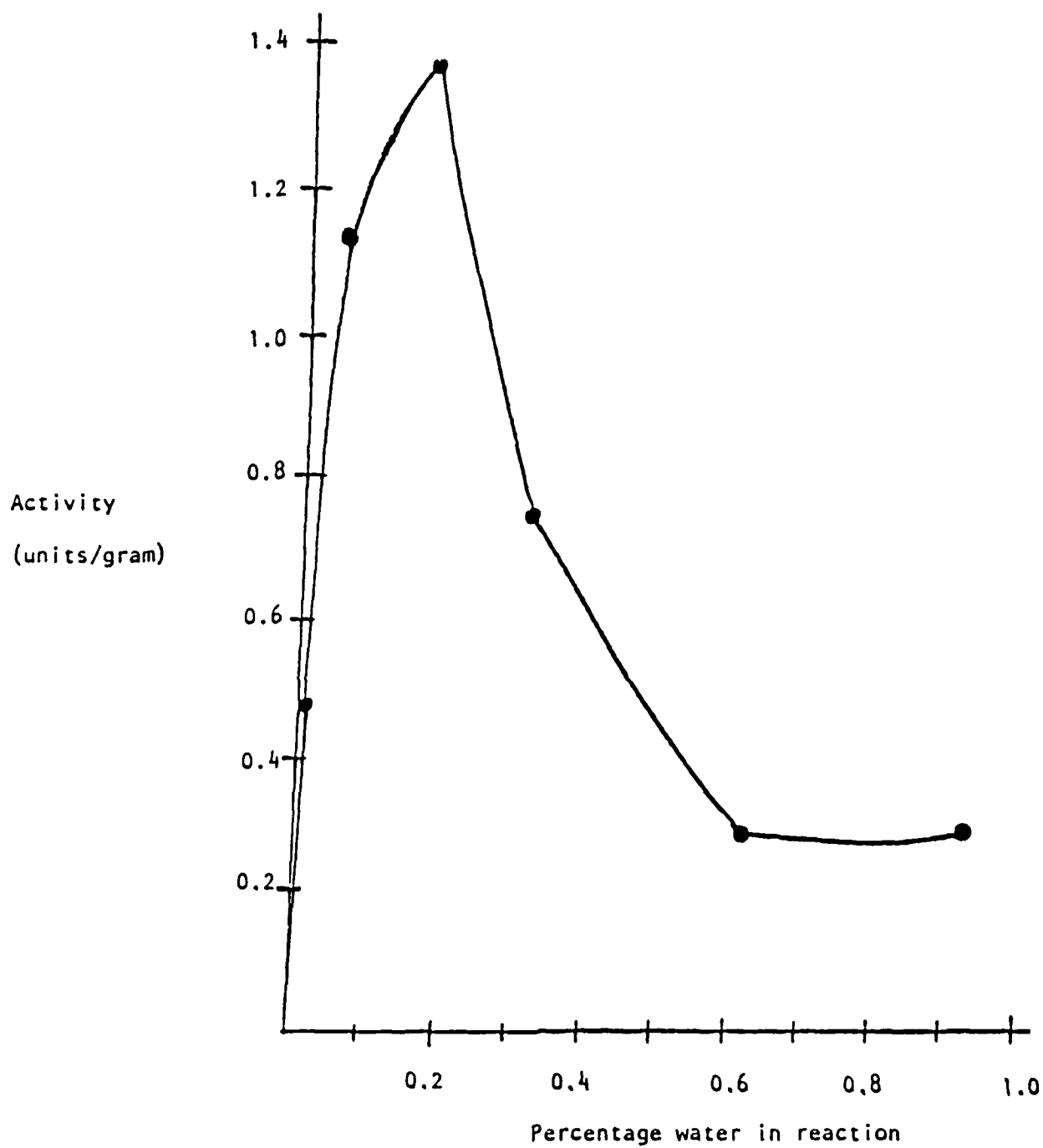


Figure 9. Specific activity of carboxyl esterase as a function percent water in the reaction mixture of beta-nitropropionate methyl ester and isopropyl alcohol

Absorbance

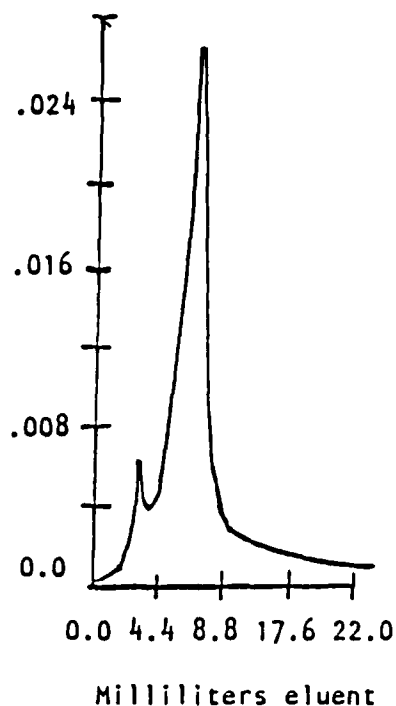


Figure 10. Initial chromatogram (0 hr) for the transesterification reaction between beta-nitropropionate methyl ester and 1-butanol in the presence of carboxyl esterase (C_{18} reverse phase column)

Absorbance

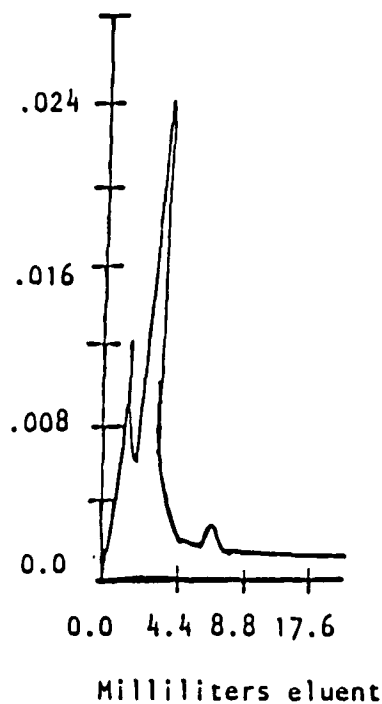


Figure 11. Chromatogram (20 hr) for the transesterification reaction between beta-nitropropionic acid methyl ester and 1-butanol in the presence of carboxyl esterase (C_{18} reverse phase column)

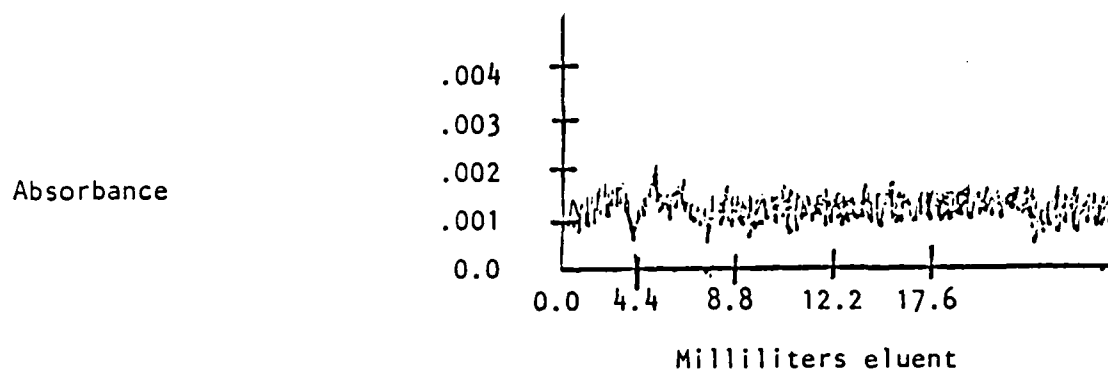


Figure 12. Chromatogram of hydrolyzed product from the transesterification reaction between cellulose and beta-nitropropionate acid methyl ester (C_{18} reverse phase column) No lipase present

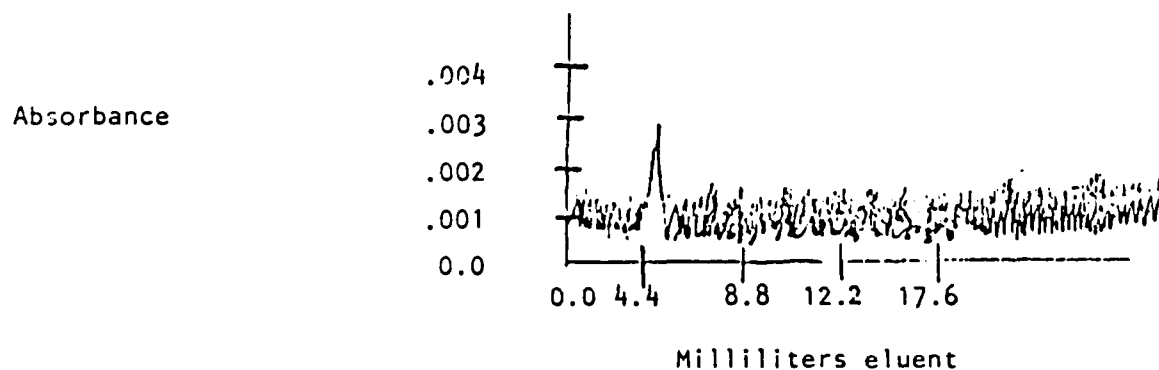


Figure 13. Chromatogram of hydrolyzed product from the transesterification reaction between cellulose and beta-nitropropionic acid methyl ester (C_{18} reverse phase column) Lipase present

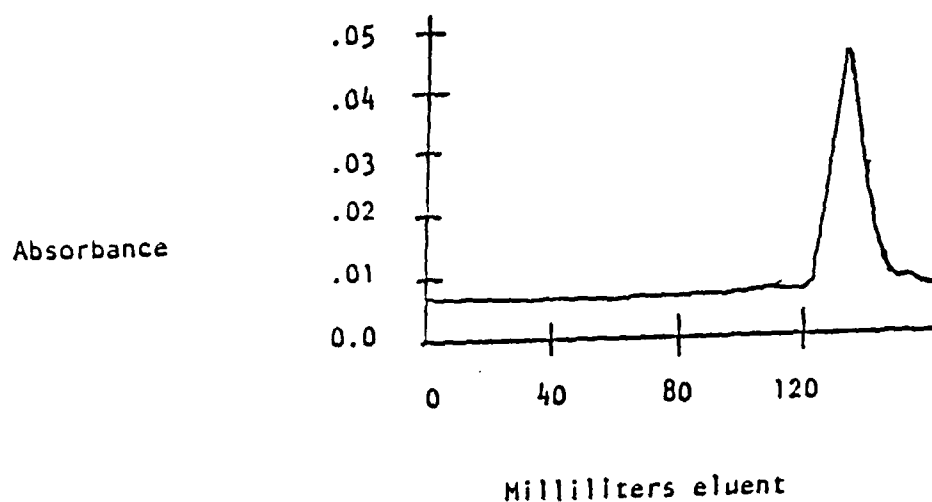


Figure 14. Chromatogram of beta-nitropropionate methyl ester (SephadexTM G-10 column)

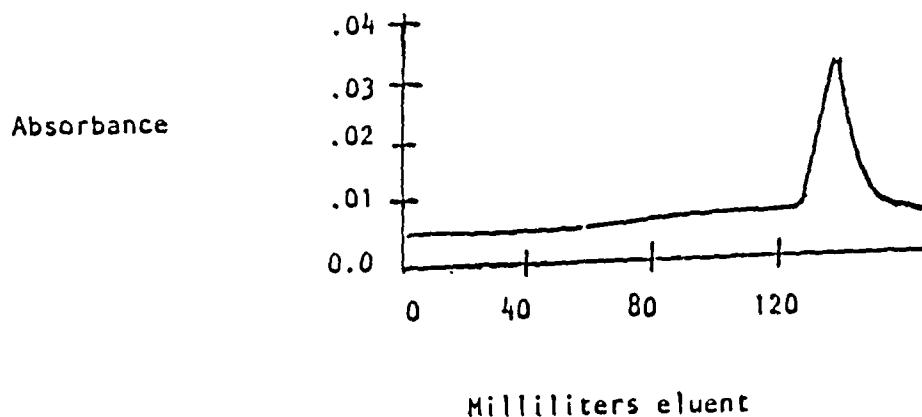


Figure 15. Chromatogram of the transesterification of beta-nitropropionic acid methyl ester and beta-methyl-D-glucoside in dioxane, with a strong acidic ion exchange resin as a catalyst (SephadexTM G-10 column)

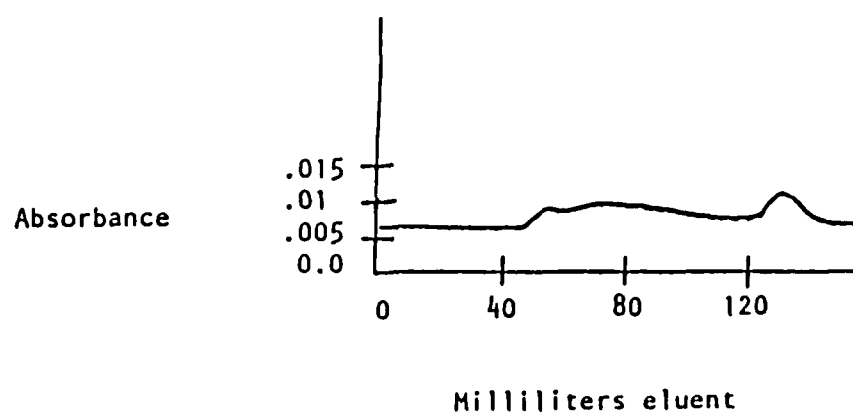


Figure 16. Chromatogram of the transesterification of beta-nitropropionic acid methyl ester and beta-methyl-D-glucoside in dioxane (SephadexTM G-10 column)

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